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Short communication

Determination of phenolic compounds in *Prunella* L. by liquid chromatography-diode array detection

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ABSTRACT

Four species of *Prunella* L. (*Prunella vulgaris* L., *Prunella laciniata* L., *Prunella grandiflora* L. and *Prunella orientalis* Bornm.) belong to the family of *Lamiaceae* and representing popular Western and Chinese herbal medicine were examined for the content of phenolic compounds. Phenolic acids (rosmarinic acid, caffeic acid, ferulic acid, chlorogenic acid, protocatechuic acid), flavonoids (rutin, quercetin) in different quantitative proportions depending on extracts were determined by the rapid, selective and accurate method combining solvent/acid hydrolysis extraction and high performance liquid chromatography-diode array detection (HPLC-DAD). Water, methanol, butanol, acetonitrile, ethyl acetate, hexane and their acidic solutions were used to examine the efficiency of different solvent systems for the extraction of phenolic compounds. Acid hydrolysis extraction was established as the most suitable extraction method for phenolic compounds.

1. Introduction

Prunella L. (*Labiatae*) is used in Western and Chinese herbal medicine, especially in the treatment of wounds. It has a wide spectrum of biological effects including anti-microbial, anti-inflammatory and immunomodulatory [1]. *Prunella* L. species exhibit high antioxidant potentials, which are tightly connected with the total phenolic content [2]. Medicinal plant *Prunella* L. contains phenolic acids (rosmarinic acid, caffeic acid, *p*-coumaric acid, chlorogenic acid) [2–4], flavonoids (kaempferol, quercetin, rutin, luteolin) [3,5] and anthocyanidins (cyanidin, delphinidin) [6]. Rosmarinic acid, the major phenolic component of the plant, has shown antioxidant, anti-inflammatory, antibacterial and anti-HIV properties [7].

Determination of phenolic compounds in plant materials should be helpful in the better explaining the complex pharmacological activity of some medicinal plants. Therefore reliable and practical methods for separation, identification and quantitative analysis of phenolic compounds in plants have been proposed. Most of the protocols are based on gas chromatography and mass spectrometry (GC–MS) [8,9], high performance liquid chromatography (HPLC), liquid chromatography and mass spectrometry (LC–MS) [10] and capillary zone electrophoresis [11]. Phenolic extracts of plant materials are always a mixture of different classes of phenolics that are soluble in the solvent system used. Most common solvents are aqueous mixtures with methanol, ethyl acetate and acetone. Furthermore, hydrolysis of glycoside bonds is often used in the extraction procedure, and thereby essential information of total phenolic compounds as aglycones can be obtained [12].

The main objective of this study was to determine phenolic acids (rosmarinic acid, caffeic acid, ferulic acid, chlorogenic acid, protocatechuic acid), flavonoids (rutin, quercetin) by high performance liquid chromatography-diode array detection (HPLC-DAD) and to examine the efficiency of different solvent systems for the extraction of phenolic compounds.

2. Materials and methods

2.1. Materials

Rosmarinic acid, quercetin hydrate, rutin and chlorogenic acid were purchased from Sigma–Aldrich (St. Louis, Missouri, USA). Caffeic acid, ferulic acid, protocatechuic acid, analytical grade of hydrochloric acid, HPLC grade of methanol, butanol, ethyl acetate, acetonitrile, hexane and formic acid were purchased from Merck (Darmstadt, Germany). All standard solutions were prepared in methanol.

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2.2. Plant materials and preparation of the plant extracts

Prunella L. species (*Prunella vulgaris* L., *Prunella laciniata* (L.) L., *Prunella grandiflora* L., *Prunella orientalis* Bornm.), were collected from four localities (Bursa, Balıkesir, Eskişehir and Antalya) in Turkey during June–July 2009. The collected samples were dried at room temperature and stored at 4 °C. The whole parts of *Prunella* L. samples (1 g) were separately blended with either water or organic solvent (methanol, butanol, ethyl acetate, acetonitrile, hexane) containing 80 mg of ascorbic acid as an antioxidant and 10 mL of 6 M HCl (final concentration 1.2 M HCl) at room temperature in dark for 8 h under magnetic stirrer. The samples were treated with nitrogen gas before extraction. The extraction was also performed with solvent extraction method using the same solvents. The hydrolysed/unhydrolysed samples (total volume 50 mL) were separated from the solid matrix by filtration through sheets of qualitative filter paper. The extracts were further passed through 0.45-μm membrane filters before HPLC-DAD analysis.

2.3. HPLC-DAD analysis

An Agilent 1200 HPLC system (Waldbronn, Germany), consisting of a vacuum degasser, binary pump, autosampler and a diode-array detector, was used. Chromatographic separations were carried out using an XBridge C18 (4.6 mm × 250 mm, i.d. 3.5 μm) column from waters. Mobile phase consists of 1% formic acid in water (solvent A) and acetonitrile (solvent B). Gradient conditions are as follows; 0–10 min 13% B, 10–20 min 41.5% B, 20–25 min 70% B, 25–35 min 10% B, total run time is 35 min. The column was equilibrated for 10 min prior to each analysis at 25 °C. Flow rate was 0.5 ml/min and injection volume was 10 μL. Data acquisition and preprocessing was done with Chemstation for LC (Agilent). The monitoring wavelength was 280 nm for protocatechuic acid and 360 nm for rosmarinic acid, quercetin, rutin, chlorogenic acid, caffeic acid, ferulic acid. Peaks were identified on the basis of comparison of retention times and UV spectra with standards of rosmarinic acid, quercetin, rutin, chlorogenic acid, caffeic acid, ferulic acid and protocatechuic acid.

3. Results and discussions

3.1. Identification of phenolic compounds in Prunella L.

Phenolic compounds were determined in four species of *Prunella* L.; *P. vulgaris* L., *P. laciniata* (L.) L., *P. grandiflora* L., *P. orientalis* Bornm., by HPLC-DAD. A chromatogram of phenolic compounds identified in *P. laciniata* L. was illustrated as an example in Fig. 1. Quercetin, rutin, rosmarinic acid, caffeic acid, ferulic acid, chlorogenic acid and protocatechuic acid were determined in pure solvent (Table 1) and acidic solvent extracts of *Prunella* L. (Table 2). Quercetin was not detected in *Prunella* L. solvent extracts except water extract of *P. grandiflora* L. However, the amount of quercetin in water extract of *P. grandiflora* L. is too little (0.36 ± 0.01 mg/g dried plant). The highest amount of quercetin was established in acidic ethyl acetate extract of *P. grandiflora* L. As can be seen from these results, the amounts of quercetin in acidic solvent extracts are higher than the amounts of quercetin in solvent extracts. Because rutin, glycoside of quercetin, can be hydrolyzed to aglycon as quercetin with strong acids and so the amounts of quercetin are higher in acid hydrolysis method (Table 2).

The amount of rutin in acidic solvent extracts was higher than those in solvent extracts. The rutin was detected in *P. vulgaris* L. samples with amounts ranged from 1.45 ± 0.06 to 4.01 ± 0.05 mg/g dried plant, *P. laciniata* (L.) L. samples with amounts ranged from 0.27 ± 0.01 to 3.12 ± 0.02 mg/g dried plant, *P. grandiflora* L. samples

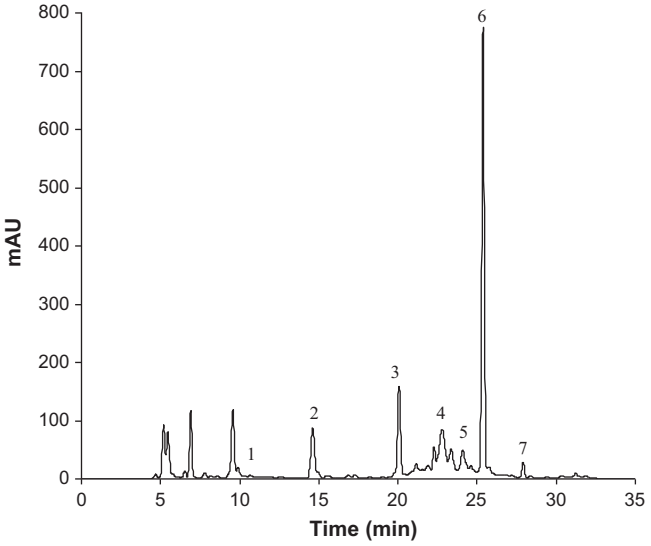

Fig. 1. The chromatogram of acidic water extract of *Prunella laciniata* L. at 280 nm (1, protocatechuic acid; 2, chlorogenic acid; 3, caffeic acid; 4, rutin; 5, ferulic acid; 6, rosmarinic acid; 7, quercetin).

with amounts ranged from 3.93 ± 0.01 to 7.13 ± 0.02 mg/g dried plant and *P. orientalis* Bornm samples with amounts ranged from 0.93 ± 0.08 to 6.52 ± 0.06 mg/g dried plant by using acid hydrolysis method. The highest amount of rutin was established in acidic butanol extract of *P. grandiflora* L. Also the amount of rutin was found in *P. vulgaris* L. to be higher than that previously reported by Cheung et al. [11].

The other phenolic compounds caffeic acid, ferulic acid, chlorogenic acid and protocatechuic acid are determined in *Prunella* L. samples at trace levels. Caffeic acid was only identified in water extract, acidic water, acidic acetonitrile and acidic ethyl acetate of *Prunella* L. except *P. grandiflora* L. Furthermore the methanol extracts of *P. grandiflora* L. contained caffeic acid. Chlorogenic acid was identified in water extracts of *Prunella* L. but not detected in *P. grandiflora* L. solvent extracts. The amount of chlorogenic acid in acidic butanol, acidic ethyl acetate, acidic water extracts ranged from 0.19 ± 0.09 to 1.07 ± 0.03 mg/g dried plant. There is no information in literature about ferulic and protocatechuic acids contents in *Prunella* L. species. Ferulic and protocatechuic acids contents were reported for the first time for *Prunella* L. extracts in our study. The highest amount of ferulic acid was established in acidic acetonitrile extract of *P. orientalis* Bornm. However, the highest amount of protocatechuic acid was found in water extract of *P. grandiflora* L.

Rosmarinic acid was predominant in all *Prunella* L. species and the amounts of rosmarinic acid ranged from 1.19 ± 0.01 to 38.28 ± 0.68 mg/g dried plant for *P. vulgaris* L., from 0.84 ± 0.01 to 39.19 ± 0.48 mg/g dried plant for *P. laciniata* (L.) L., from 0.70 ± 0.01 to 36.45 ± 0.04 mg/g dried plant for *P. grandiflora* L. and from 0.24 ± 0.01 to 18.95 ± 0.14 for *P. orientalis* Bornm. Rosmarinic acid content was found in *P. vulgaris* L. to be higher than that previously reported by Cheung et al. [11]. This difference could be explained by different location, analytical method used and extraction procedure. Rosmarinic acid is the main antioxidant constituent in the *Lamiaceae* family [13]. As rosmarinic acid is known antiviral, antibacterial, antioxidant and anti-inflammatory [7], caffeic acid was found to have high activity comparable to that of quercetin [14] and ferulic acid was shown to inhibit the photoperoxidation of linoleic acid at high concentrations [15]. *Prunella* L. extracts may have antioxidant and anti-inflammatory properties due to quercetin, rutin, chlorogenic and protocatechuic acids contents [16,17]. On the other hand, phenolic compounds have not

Table 1
The amounts of phenolic compounds extracted from *Prunella* L. by using solvent extraction (milligrams per gram dried plant).

	QU	RU	RA	CA	CHA	FA	PCA	Total
PVW	nd	0.61 ± 0.01	9.46 ± 0.03	0.29 ± 0.01	0.32 ± 0.01	nd	nd	10.68 ± 0.04
PVM	nd	1.27 ± 0.05	21.85 ± 0.45	nd	nd	nd	nd	23.12 ± 0.45
PVB	nd	0.10 ± 0.01	1.19 ± 0.01	nd	nd	nd	nd	1.29 ± 0.01
PVA	nd	nd	2.32 ± 0.01	nd	nd	nd	nd	2.32 ± 0.01
PVE	nd	0.07 ± 0.01	2.78 ± 0.01	nd	nd	nd	nd	2.85 ± 0.01
PVH	nd	nd	nd	nd	nd	nd	nd	nd
PLW	nd	0.76 ± 0.01	18.81 ± 0.17	0.26 ± 0.01	0.45 ± 0.01	2.04 ± 0.02	nd	22.32 ± 0.17
PLM	nd	1.01 ± 0.03	17.94 ± 0.42	nd	nd	nd	nd	18.95 ± 0.42
PLB	nd	0.12 ± 0.01	1.31 ± 0.01	nd	nd	nd	nd	1.43 ± 0.01
PLA	nd	nd	1.28 ± 0.01	nd	nd	nd	nd	1.28 ± 0.01
PLE	nd	nd	0.84 ± 0.01	nd	nd	nd	nd	0.84 ± 0.01
PLH	nd	nd	nd	nd	nd	nd	nd	nd
PGW	0.36 ± 0.01	1.53 ± 0.02	15.24 ± 0.18	nd	nd	nd	0.11 ± 0.01	17.23 ± 0.18
PGM	nd	6.74 ± 0.14	21.83 ± 0.38	0.11 ± 0.01	nd	0.21 ± 0.01	nd	28.88 ± 0.41
PGB	nd	0.22 ± 0.01	1.07 ± 0.01	nd	nd	nd	nd	1.29 ± 0.01
PGA	nd	nd	0.70 ± 0.01	nd	nd	nd	nd	0.70 ± 0.01
PGE	nd	0.14 ± 0.01	1.93 ± 0.01	nd	nd	nd	nd	2.07 ± 0.01
PGH	nd	nd	nd	nd	nd	nd	nd	nd
POW	nd	0.45 ± 0.01	5.42 ± 0.05	0.39 ± 0.01	0.14 ± 0.01	1.26 ± 0.01	0.08 ± 0.01	7.71 ± 0.05
POM	nd	0.99 ± 0.01	9.63 ± 0.11	nd	nd	0.41 ± 0.01	nd	11.02 ± 0.11
POB	nd	0.06 ± 0.01	0.91 ± 0.01	nd	nd	nd	nd	0.97 ± 0.01
POA	nd	nd	0.83 ± 0.01	nd	nd	nd	nd	0.83 ± 0.01
POE	nd	nd	0.24 ± 0.01	nd	nd	nd	nd	0.24 ± 0.01
POH	nd	nd	nd	nd	nd	nd	nd	nd

Mean of two determinations ± SD. *nd* not detected, *QU* quercetin, *RU* rutin, *RA* rosmarinic acid, *CA* caffeic acid, *CHA* chlorogenic acid, *FA* ferulic acid, *PCA* protocatechuic acid, *PV Prunella vulgaris* L., *PL Prunella laciniata* L., *PO Prunella orientalis* Bornm., *PG Prunella grandiflora* L., *W* water, *M* methanol, *B* butanol, *A* acetonitrile, *E* ethyl acetate, *H* hexane, *SD* standard deviation.

been identified in hexane and acidic hexane extracts of *Prunella* L.

3.2. Validation of analytical method

The linearity of the HPLC-DAD method was investigated for phenolic compounds in the range 1–100 mg/L at fourteen concentration levels. Calibration plots with correlation coefficient $R^2 \geq 0.998$ were obtained by reporting peak areas as a function of phenolic compounds concentrations (Table 3). The validation of the quantitative determination of phenolic compounds in *Prunella* L. samples was performed by limits of detection (LOD, 3 s/m), limits of quantification (LOQ, 10 s/m) and recovery (%) of each phenolic compound (Table 3). Where *s* is the sample standard deviation for the replicates and *m* is the slope of the calibration curve. LOD ranged from 0.07 to 0.14 mg/L and LOQ ranged from 0.23 to 0.47 mg/L for 7 phenolic compounds. The extraction efficiency of the phenolic standards of quercetin, rutin, rosmarinic acid, caffeic acid, ferulic acid, chlorogenic acid and protocatechuic acid from *P. vulgaris* L. sample was evaluated by spiking the mixture of phenolic compounds to samples and extracted using methanol and acidic methanol solvents.

Table 2
The amounts of phenolic compounds extracted from *Prunella* L. by using acid hydrolysis (milligrams per gram dried plant).

	QU	RU	RA	CA	CHA	FA	PCA	Total
PVAW	0.19 ± 0.01	2.29 ± 0.01	9.53 ± 0.05	0.91 ± 0.02	nd	0.25 ± 0.01	0.07 ± 0.01	13.23 ± 0.06
PVAM	1.70 ± 0.01	3.88 ± 0.09	4.55 ± 0.05	nd	nd	nd	nd	10.14 ± 0.10
PVAB	0.79 ± 0.01	4.01 ± 0.05	5.69 ± 0.08	nd	0.52 ± 0.02	nd	nd	11.01 ± 0.10
PVAA	2.10 ± 0.14	nd	38.28 ± 0.68	0.47 ± 0.01	nd	nd	nd	40.85 ± 0.69
PVAE	2.10 ± 0.26	1.45 ± 0.06	13.32 ± 0.67	0.73 ± 0.05	0.19 ± 0.09	nd	nd	17.79 ± 0.73
PVAH	nd	nd	nd	nd	nd	nd	nd	nd
PLAW	0.72 ± 0.01	2.04 ± 0.04	13.17 ± 0.22	1.61 ± 0.02	0.34 ± 0.01	0.70 ± 0.01	0.06 ± 0.01	18.63 ± 0.23
PLAM	2.92 ± 0.05	2.49 ± 0.11	4.32 ± 0.11	nd	nd	nd	nd	9.73 ± 0.16
PLAB	2.30 ± 0.07	3.12 ± 0.02	5.50 ± 0.01	nd	0.82 ± 0.01	nd	nd	11.74 ± 0.07
PLAA	3.52 ± 0.07	nd	39.19 ± 0.48	0.64 ± 0.01	nd	nd	nd	43.36 ± 0.49
PLAE	4.31 ± 0.02	0.27 ± 0.01	11.78 ± 0.06	0.90 ± 0.01	nd	nd	nd	17.26 ± 0.07
PLAH	nd	nd	nd	nd	nd	nd	nd	nd
PGAW	1.09 ± 0.06	3.96 ± 0.13	8.79 ± 0.29	1.82 ± 0.05	nd	0.56 ± 0.01	0.09 ± 0.01	16.31 ± 0.03
PGAM	5.51 ± 0.23	3.93 ± 0.01	3.49 ± 0.02	1.13 ± 0.03	nd	nd	nd	14.07 ± 0.23
PGAB	5.24 ± 0.03	7.13 ± 0.02	5.71 ± 0.03	nd	1.07 ± 0.03	nd	nd	19.15 ± 0.06
PGAA	5.18 ± 0.01	nd	36.45 ± 0.04	1.80 ± 0.01	nd	nd	nd	43.43 ± 0.04
PGAE	6.41 ± 0.11	nd	12.99 ± 0.21	1.30 ± 0.05	nd	nd	0.08 ± 0.01	20.77 ± 0.24
PGAH	nd	nd	nd	nd	nd	nd	nd	nd
POAW	0.31 ± 0.01	1.06 ± 0.03	6.46 ± 0.18	1.14 ± 0.04	nd	0.45 ± 0.01	nd	9.43 ± 0.19
POAM	1.56 ± 0.11	0.93 ± 0.08	2.00 ± 0.27	nd	nd	nd	nd	4.49 ± 0.30
POAB	1.65 ± 0.01	1.98 ± 0.01	3.55 ± 0.04	nd	0.65 ± 0.01	nd	nd	7.82 ± 0.04
POAA	1.58 ± 0.14	6.52 ± 0.06	18.95 ± 0.14	0.82 ± 0.03	nd	2.79 ± 0.08	nd	30.67 ± 0.22
POAE	1.74 ± 0.02	2.24 ± 0.02	6.92 ± 0.05	0.74 ± 0.01	nd	nd	nd	11.64 ± 0.06
POAH	nd	nd	nd	nd	nd	nd	nd	nd

Mean of two determinations ± SD. *nd* not detected, *QU* quercetin, *RU* rutin, *RA* rosmarinic acid, *CA* caffeic acid, *CHA* chlorogenic acid, *FA* ferulic acid, *PCA* protocatechuic acid, *PV Prunella vulgaris* L., *PL Prunella laciniata* L., *PO Prunella orientalis* Bornm., *PG Prunella grandiflora* L., *AW* acidic water, *AM* acidic methanol, *AB* acidic butanol, *AA* acidic acetonitrile, *AE* acidic ethyl acetate, *AH* acidic hexane, *SD* standard deviation.

Table 3
Validation parameters and recovery of the phenolic compounds in *Prunella vulgaris* L. extracts.

Phenolic compounds	LOD (mg L^{-1})	LOQ (mg L^{-1})	R^2	Recovery (%)	
				Extraction	Acid hydrolysis
Quercetin	0.07	0.23	0.999	102 ± 2	99 ± 2
Rutin	0.11	0.36	0.998	82 ± 3	51 ± 1
Rosmarinic acid	0.08	0.27	0.999	95 ± 2	91 ± 4
Caffeic acid	0.08	0.26	0.999	88 ± 4	74 ± 3
Chlorogenic acid	0.13	0.44	0.998	90 ± 4	87 ± 4
Ferulic acid	0.14	0.47	0.999	102 ± 2	102 ± 1
Protocatechuic acid	0.13	0.43	0.999	94 ± 4	99 ± 1

LOD limits of detection, *LOQ* limits of quantification.

The recovery study was carried out only for the phenolic compounds identified by HPLC-DAD. The mean percentage recoveries ranged from 82 ± 3 (rutin) to 102 ± 2% (quercetin, ferulic acid) for solvent extraction method and ranged from 51 ± 1 (rutin) to 102 ± 1% (ferulic acid) for acid hydrolysis method. Similar recoveries were obtained for solvent extraction and acid hydrolysis method except for rutin. The least recovery was obtained as 51 ± 1% for rutin in acid hydrolysis method, but 82 ± 3% in solvent extraction method. Rutin, one of the glycoside of quercetin, is hydrolyzed in acid hydrolysis. Therefore, the recovery of solvent extraction method is higher than the recovery of acid hydrolysis method for rutin. However, the concentration of quercetin extracted by using acid hydrolysis is higher than the concentration of quercetin extracted by using solvent extraction in *Prunella* L. samples. The acid hydrolysis is particularly suitable for the flavonoid extraction because many phenolic compounds occurring as glycosides or esters. All other recoveries are in experimental error range. In the calculation of the final results, the recoveries of the pure phenolic standards were taken into account.

4. Conclusions

Extraction efficiency of different solvent systems for extracting the phenolic compounds in *Prunella* L. species were examined. Phenolic compounds (rosmarinic acid, caffeic acid, ferulic acid, chlorogenic acid, protocatechuic acid, rutin and quercetin) were determined in *Prunella* L. samples by HPLC-DAD. The phenolic compounds were quantified in different proportions depending on extraction methods. Acid hydrolysis extraction was found as the most suitable extraction method for the phenolic compounds. Acidic acetonitrile was the most efficient solvent for extracting phenolic compounds in *Prunella* L. species. Rosmarinic acid was the most abundant phenolic compound determined in all *Prunella* L. samples.

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